SUPPLEMENTARY APPENDIX

Cytomegalovirus shapes long-term immune reconstitution after allogeneic stem cell transplantation

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Supplementary Material

Supplementary Methods

Patients

Immune reconstitution was monitored as part of routine immunological follow-up in all patients undergoing allogeneic stem cell transplantation at our center between 2005 and 2009. Blood samples were collected prior to transplant conditioning (roughly one month) and at 3, 6, 12 and 24 months after transplant as part of routine clinical practice. As previously reported (Corre et al., 2010; Moins-Teisserenc et al., 2013; Servais et al., 2014), all patients provided written informed consent for use of protected health data for research, in accordance with the Declaration of Helsinki. The present study focuses on patients with immune reconstitution profiling available at twelve months from transplant, regardless of the indication for HSCT, source of transplantable cells, and of conditioning regimen before HSCT. One hundred ninety patients met these criteria. Within this cohort, reconstitution monitoring at all other study points (pre-transplant, and after 3, 6 and 24 months post-transplant) was available in 77 patients. Kinetics of reconstitution was studied in this smaller cohort. Characteristics of both cohorts are recapitulated in **Table 1**.

Definition of clinical variables

Myeloablative conditioning regimens and reduced-intensity conditioning were defined as previously described (Bacigalupo et al., 2009). Details on conditioning regimens are provided for the twelve-month cohort in **Supplementary Table 2**. Acute and chronic GVHD were scored according to modified Glucksberg criteria (Przepiorka et al., 1995) and to the Committee of the International Bone Marrow Transplant Registry (CIBMTR) Consensus Criteria used in 2005 (Atkinson et al., 1989), respectively. CMV replication was defined as one or more quantitative PCR > 1000 copies/mL prompting preemptive treatment as previously published (Gouarin et al., 2007; Kheav et al., 2014; Schnepf et al., 2013). Policies regarding prophylaxes and treatments of infections, notably CMV reactivation, acute and chronic graft-versus-host disease were in accordance with European Bone

Marrow Transplantation (EBMT) recommendations as previously reported (Corre et al., 2010; Servais et al., 2014).

Controls

Thirty two blood samples from healthy donors (HD) were collected after informed consent from the local blood donor center (Etablissement Français du Sang, Hôpital Saint-Louis).

Flow cytometry

Absolute lymphocyte count was calculated from freshly collected blood using the TruCount system (Becton Dickinson, le Pont de la Claix, France) with CD3-APC, CD45-PerCP-Cy5.5, CD8FITC, and CD4PE mAbs. A minimum of 10,000 lymphocytes were analyzed and isotype-matched controls were performed in all cases. Lymphocyte subpopulations were labeled with the following monoclonal antibodies (all from BD Bioscience): anti-CD3-FITC, -CD4-PE, -CD8-PercP, -CD25-PECy7, -HLA-DR-APC, -CD45RA-PECy7, -CD45RO APC-Cy7, -CCR7 APC, -CD62L-APC, -CD127-PECy7, CD28-PECy7, -CD16-PE, -CD56-PercP, CD5-FITC, CD19-PE, CD27-PercP. Sample acquisition was performed using a FACSCanto II™ flow cytometer and data were analyzed using FACS Diva™ (BD Biosciences). Analyzed populations are summarized in **Table 2**. Regulatory T cell populations were defined based on the CD4+/CD25+/CD127low phenotype as previously published (Liu et al., 2006; Xhaard et al., 2014). Only the restricted panel was assessed in the control samples, whereas the extended panel was used for patient samples.

Statistical analyses

All lymphocyte subsets were studied as proportions of the patient's total lymphocyte count at the time of sampling. Information was missing on a median of 2/25 subsets (range 0-4) in 32/190 (17%) patients from the global cohort. Imputation of missing data was performed with the K-nearest neighbors' method (Troyanskaya et al., 2001).

Because the resulting datasets contained frequencies of immune subsets, they can be viewed as contingency tables. Multivariate analysis was thus performed by correspondence analysis (CA), which is conceptually analogous to principal component analysis (PCA), but is more adapted to contingency tables (Husson et al., 2010). Briefly, CA, like PCA, allows description of highly multidimensional data by projecting data on orthogonal dimensions containing a maximum amount of information. This reduces the number of variables studied with limited distortion of the initial dataset. Dimensions are sequentially ordered with the first dimension containing the maximal amount of information (proportion of variance). 'Between-group analysis' was used to perform supervised clustering of CA data (Culhane et al., 2002).

Similarity between patients' and controls' datasets was evaluated with the 'RV' coefficient, which measures the relationship of two sets of variables defined for the same individuals. Significance of dissimilarity between sets was tested with a permutation test (Josse et al., 2008).

Unsupervised clustering was performed using correlation as distance and Ward's method for cluster identification (Meyniel et al., 2010). Approximately unbiased tests were used to detect statistically significant clusters with $P<.05$ (Shimodaira, 2004). Of note, the heatmap of immune populations according to CMV serostatus was performed after quantile normalization of data for graphical purpose. Datasets were otherwise not normalized.

One-way analysis of variance according to categorical variables corresponding to pre-transplant characteristics and post-transplant events (censoring those events at the 12-month study point) was performed with the non-parametric Kruskall-Wallis test. Resulting *P* values were adjusted for multiple testing by the Benjamini–Hochberg method.(Benjamini and Hochberg, 1995) When two variables were each significantly (adjusted $P<.05$) impacting a single dimension score, two-way analysis of variance (ANOVA) accounting for interaction was performed. For that purpose, CMV serostatus was considered as an ordinal variable (in the following order: Donor[D]-/Recipient[R]-, D+/R-, D+/R+, D-/R+).

Non-relapse mortality (NRM) and cumulative incidence of relapse (CIR) were defined with a landmark at 12 months, corresponding to the time of immune reconstitution assessment, considering relapse and death without relapse as competing events. Multivariate Fine & Gray models were established after limited backward selection (Fine and Gray, 1999).

All analyses were carried with R 3.0.2 (www.cran.r-project.org) using packages *impute*, *preprocessCore*, *Made4*, *FactoMineR*, *cmprsk*, and *pvclust*.

Supplementary References

Atkinson, K., Horowitz, M.M., Gale, R.P., Lee, M.B., Rimm, A.A., and Bortin, M.M. (1989). Consensus among bone marrow transplanters for diagnosis, grading and treatment of chronic graft-versus-host disease. Committee of the International Bone Marrow Transplant Registry. Bone Marrow Transplant *4*, 247-254.

Bacigalupo, A., Ballen, K., Rizzo, D., Giralt, S., Lazarus, H., Ho, V., Apperley, J., Slavin, S., Pasquini, M., Sandmaier, B.M.*, et al.* (2009). Defining the intensity of conditioning regimens: working definitions. Biol Blood Marrow Transplant *15*, 1628-1633.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. J Roy Stat Soc B Met *57*, 289-300.

Corre, E., Carmagnat, M., Busson, M., de Latour, R.P., Robin, M., Ribaud, P., Toubert, A., Rabian, C., and Socie, G. (2010). Long-term immune deficiency after allogeneic stem cell transplantation: B-cell deficiency is associated with late infections. Haematologica *95*, 1025-1029.

Culhane, A.C., Perriere, G., Considine, E.C., Cotter, T.G., and Higgins, D.G. (2002). Between-group analysis of microarray data. Bioinformatics *18*, 1600-1608.

Fine, J.P., and Gray, R.J. (1999). A Proportional Hazards Model for the Subdistribution of a Competing Risk. Journal of the American Statistical Association *94*, 496-509.

Gouarin, S., Vabret, A., Scieux, C., Agbalika, F., Cherot, J., Mengelle, C., Deback, C., Petitjean, J., Dina, J., and Freymuth, F. (2007). Multicentric evaluation of a new commercial cytomegalovirus real-time PCR quantitation assay. J Virol Methods *146*, 147-154.

Husson, F., Le, S., and Pages, J. (2010). Exploratory Multivariate Analysis by Example Using R (Boca Raton, Chapman & Hall/CRC).

Josse, J., Pag\, J., \#232, and Husson, F. (2008). Testing the significance of the RV coefficient. Comput Stat Data Anal *53*, 82-91.

Kheav, V.D., Busson, M., Scieux, C., Peffault de Latour, R., Maki, G., Haas, P., Mazeron, M.C., Carmagnat, M., Masson, E., Xhaard, A.*, et al.* (2014). Favorable impact of natural killer cell reconstitution on chronic graft-versus-host disease and cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation. Haematologica
.

Liu, W., Putnam, A.L., Xu-Yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., Fazekas de St Groth, B.*, et al.* (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med *203*, 1701-1711.

Meyniel, J.P., Cottu, P.H., Decraene, C., Stern, M.H., Couturier, J., Lebigot, I., Nicolas, A., Weber, N., Fourchotte, V., Alran, S.*, et al.* (2010). A genomic and transcriptomic approach for a differential diagnosis between primary and secondary ovarian carcinomas in patients with a previous history of breast cancer. BMC Cancer *10*, 222.

Moins-Teisserenc, H., Busson, M., Herda, A., Apete, S., Peffault de Latour, R., Robin, M., Xhaard, A., Toubert, A., and Socie, G. (2013). CD19+CD5+ B cells and B1-like cells following allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant *19*, 988-991.

Przepiorka, D., Weisdorf, D., Martin, P., Klingemann, H.G., Beatty, P., Hows, J., and Thomas, E.D. (1995). 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant *15*, 825-828.

Schnepf, N., Scieux, C., Resche-Riggon, M., Feghoul, L., Xhaard, A., Gallien, S., Molina, J.M., Socie, G., Viglietti, D., Simon, F.*, et al.* (2013). Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV assay in the era of the CMV international standard. J Clin Microbiol *51*, 2096-2102.

Servais, S., Lengline, E., Porcher, R., Carmagnat, M., Peffault de Latour, R., Robin, M., Sicre de Fontebrune, F., Clave, E., Maki, G., Granier, C.*, et al.* (2014). Long-Term Immune Reconstitution and Infection Burden after Mismatched Hematopoietic Stem Cell Transplantation. Biol Blood Marrow Transplant.

Shimodaira, H. (2004). Approximately unbiased tests of regions using multistep-multiscale bootstrap resampling. Annals of Statistics *32*, 2616-2641.

Troyanskaya, O., Cantor, M., Sherlock, G., Brown, P., Hastie, T., Tibshirani, R., Botstein, D., and Altman, R.B. (2001). Missing value estimation methods for DNA microarrays. Bioinformatics *17*, 520-525.

Xhaard, A., Moins-Teisserenc, H., Busson, M., Robin, M., Ribaud, P., Dhedin, N., Abbes, S., Carmagnat, M., Kheav, V.D., Maki, G.*, et al.* (2014). Reconstitution of regulatory T-cell subsets after allogeneic hematopoietic SCT. Bone Marrow Transplant *49*, 1089-1092.

Supplementary Table 1. Comparison of study populations to the global transplant population

	Overall transplant population (N=410)		12-month cohort (N=190)			Longitudinal cohort (N=77)		
	N	%	N	%	*P=**	N	%	*P=**
Recipient Age, years (median, range)	39	(5 - 68)	38	(5 - 66)	NS	40	(8 - 64)	0.06
0 - 18 years	64	16%	26	14%		9	12%	
18 - 45 years	189	46%	91	48%		39	50%	
> 45 years	157	38%	73	38%		29	38%	
Diagnosis					NS			NS
Acute leukemia (AML/ALL)	188	46%	85	45%		40	52%	
Other	222	54%	105	55%		37	48%	
Gender Matching					NS			NS
Female to Male	97	24%	43	23%		14	18%	
Other	265	64%	129	68%		58	76%	
NA	48	12%	18	9%		5	6%	
Donor Age, years (median, range)	35	(0 - 62)	35	(0 - 62)	0.06	35	(0 - 62)	NS
0 - 18 years	71	17%	29	15%		7	9%	
18 - 45 years	212	52%	96	51%		49	64%	
> 45 years	92	22%	53	28%		16	21%	
NA	35	9%	12	6%		5	6%	
Stem cell Source					NS			NS
Bone Marrow	123	30%	63	33%		26	34%	
Peripheral Blood	240	59%	110	58%		46	60%	
Cord Blood	47	11%	17	9%		5	6%	
Donor matching					0.06			0.08
Matched sibling	194	48%	100	53%		46	60%	
Matched unrelated donor	133	32%	52	27%		19	25%	
Mismatched unrelated donor	38	9%	21	11%		7	9%	
Cord Blood	45	11%	17	9%		5	6%	
Conditioning Regimen					NS			NS
Reduced Intensity	212	52%	99	52%		37	48%	
Myeloablative	198	48%	91	48%		40	52%	
Antithymocyte Globulins					NS			0.06
Yes	111	27%	46	24%		14	18%	
No	299	83%	144	86%		63	82%	
Total Body Irradiation					NS			NS
Yes	183	45%	82	43%		28	36%	
No	227	55%	108	57%		49	64%	
CMV Serostatus (D/R)					0.02			0.09
Negative / Negative	120	29%	53	28%		25	32%	
Positive / Negative	53	13%	46	24%		16	21%	
Negative / Positive	106	26%	35	18%		15	20%	
Positive / Positive	127	31%	53	28%		21	27%	
NA	4	1%	3	2%		0	0%	

*compared to the Global population; Mann-Whitney's, Fisher's, and Kruskal-Wallis tests for continuous, dichotomic, and ordinal (donor matching, CMV serostatus) variables

NS: Non-significant (P>0.05)

Supplementary Table 2. Conditioning Regimens of the twelve-month cohort (N=190)

Conditioning Regimen	N	%
Myeloablative	91	48%
BuCy±ATG	56	29%
Bu/Mel±Flu	3	1%
TBI/Cy±Flu±ATG	30	16%
TBI/Mel	1	1%
Bu/Flu	1	1%
		0
Reduced Intensity	99	52%
TBI	18	9%
TBI/Flu±ATG	26	14%
TBI/Cy±Flu±ATG	7	4%
Flu/Bu±ATG	20	11%
Flu/Bu/Cy±ATG	3	1%
Cy±Flu±ATG	4	2%
Flu/Mel±ATG	21	11%

Supplementary Table 3

Two-way ANOVA of clinical factors and first (DIM1) and second (DIM2) dimensions of variance of immune patterns

DIM1	**F**	***P*=**
CMV Serostatus	61.581	3.44E-13
CMV Reactivation	18.172	3.23E-05
Interaction Term	4.319	0.0391
DIM2	**F**	***P*=**
Lymphopenia	52.705	1.12E-11
Chronic GVHD	8.304	0.00444
Interaction Term	0.398	0.529

Supplementary Figures 1-5.

Supplementary Figure 1. Boxplot of immune subsets as fractions of total lymphocyte count; Frequencies of immune subsets are displayed on a $\log_{10}$ scale. Notches indicate median values.

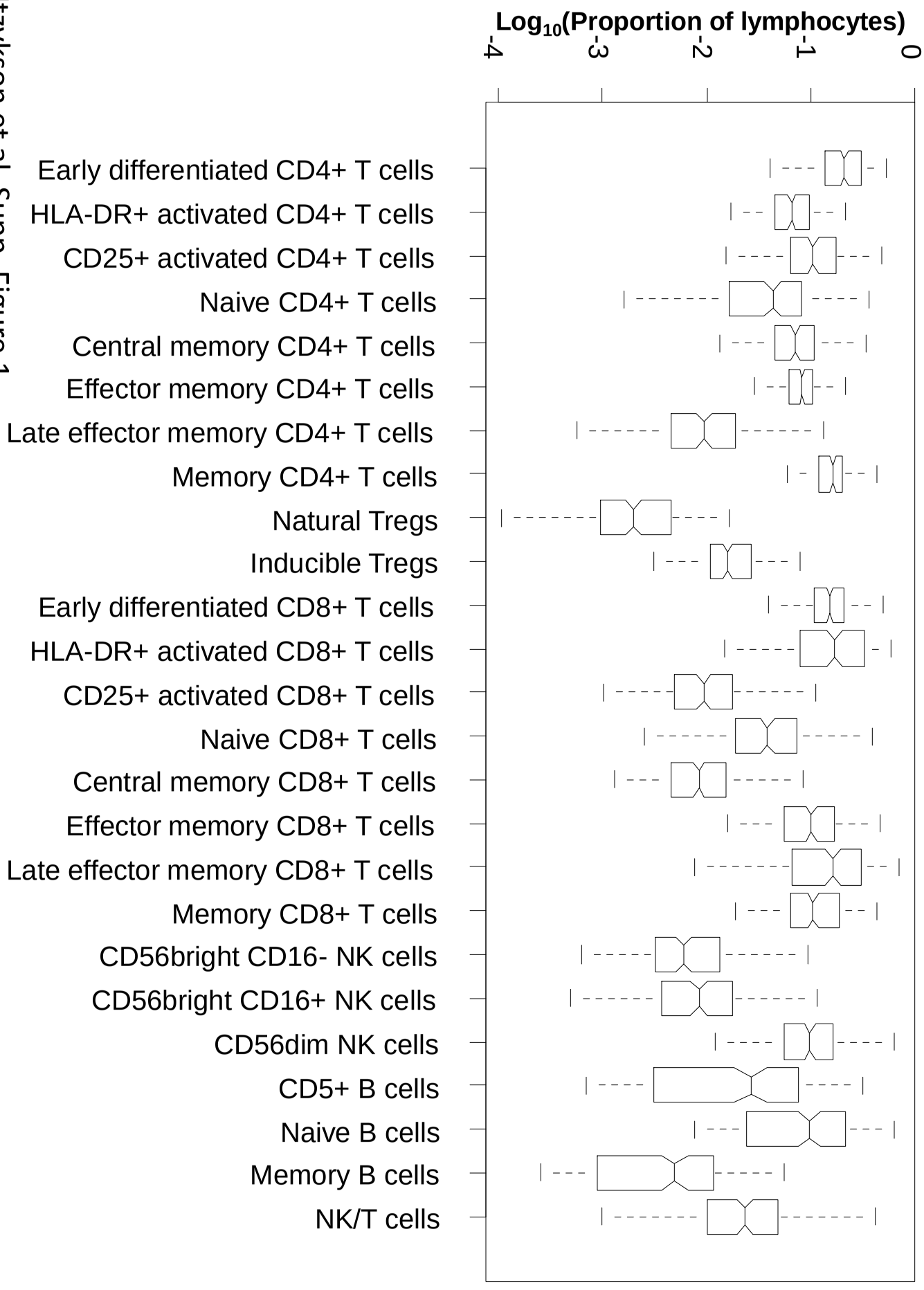
Supplementary Figure 2. Dendrogram of the 25 immune populations in 190 patients at 12 months from transplant after unsupervised clustering by Ward method of correlation distances. Red rectangles indicate clusters with significance level of $P< .05$ (approximately unbiased tests(Shimodaira, 2004)).

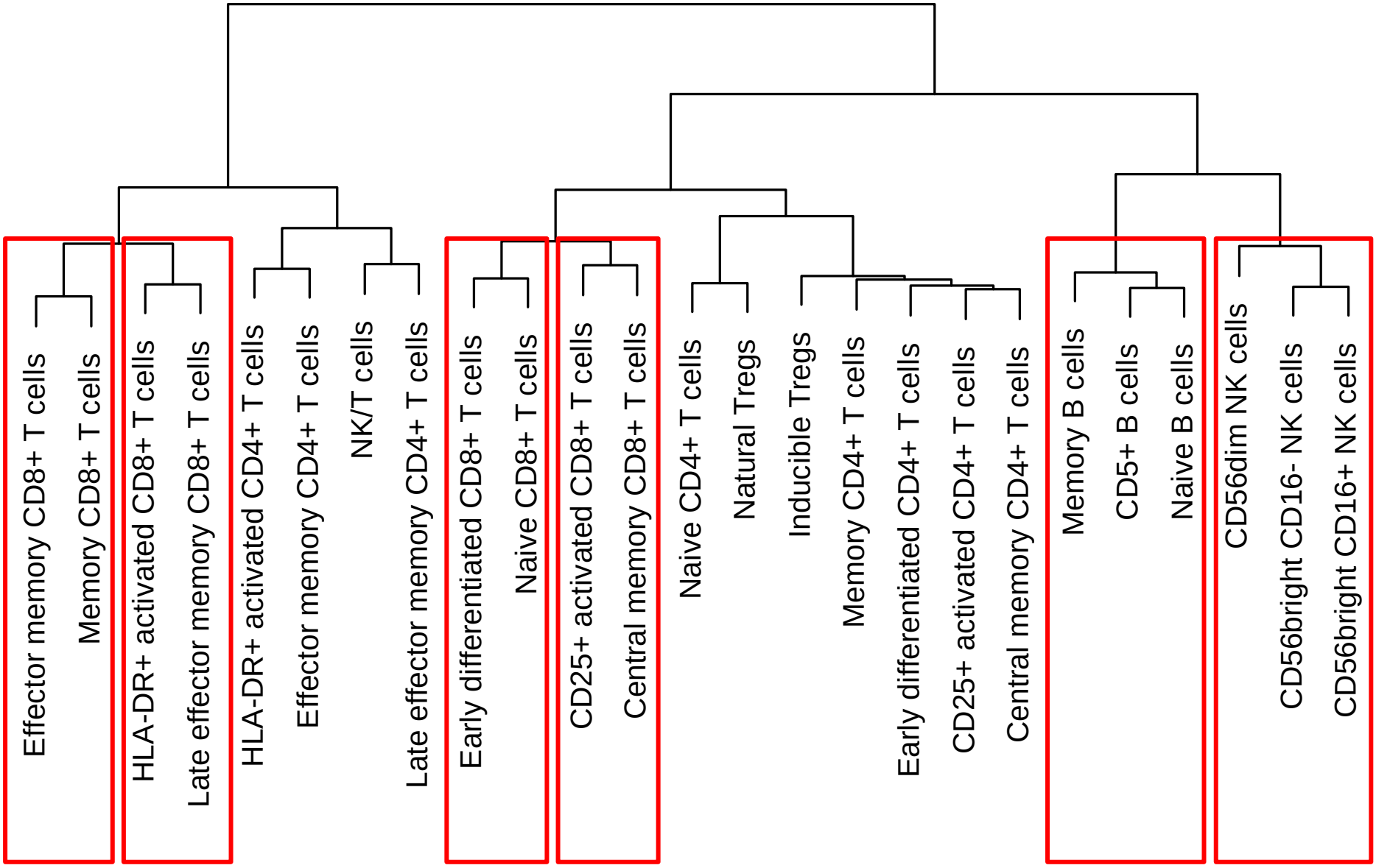
Supplementary Figure 3. Matrix of log-transformed P values of Kruskall-Wallis tests for one-way analysis of variance between each of the first five dimensions from the correspondence analysis of immune pattern analyzed as absolute number of lymphocyte populations on 190 patients at 12 months from transplant. Analyzed variables are similar to **Figure 3**. The variables are ordered according to a dendrogram resulting from unsupervised clustering of explanatory variables.

Supplementary Figure 4. Linear correlation between the number of CMV replication episodes and DIM1. Red bars indicate median values of DIM1 for each group of patients clustered according to the number of CMV replication episodes.

Supplementary Figure 5. Plots of the first 3 dimensions (DIM1-3) from the correspondence analysis of immune pattern, determined on a panel of 25 lymphocyte subsets (indicated in Table 2) in 190 patients with 12 months after transplant, according to **A.** recipient's CMV serostatus (negative: red dots, positive: blue dots), **B.** donor's CMV serostatus (negative: red dots, positive: blue dots), **C.** combination of Donor (D)/Recipient (R) CMV serostatus (D-/R-: red dots, other combinations: blue dots), **D.** CMV reactivation in the first 12 months after transplant (absent: red dots, present: blue dots).

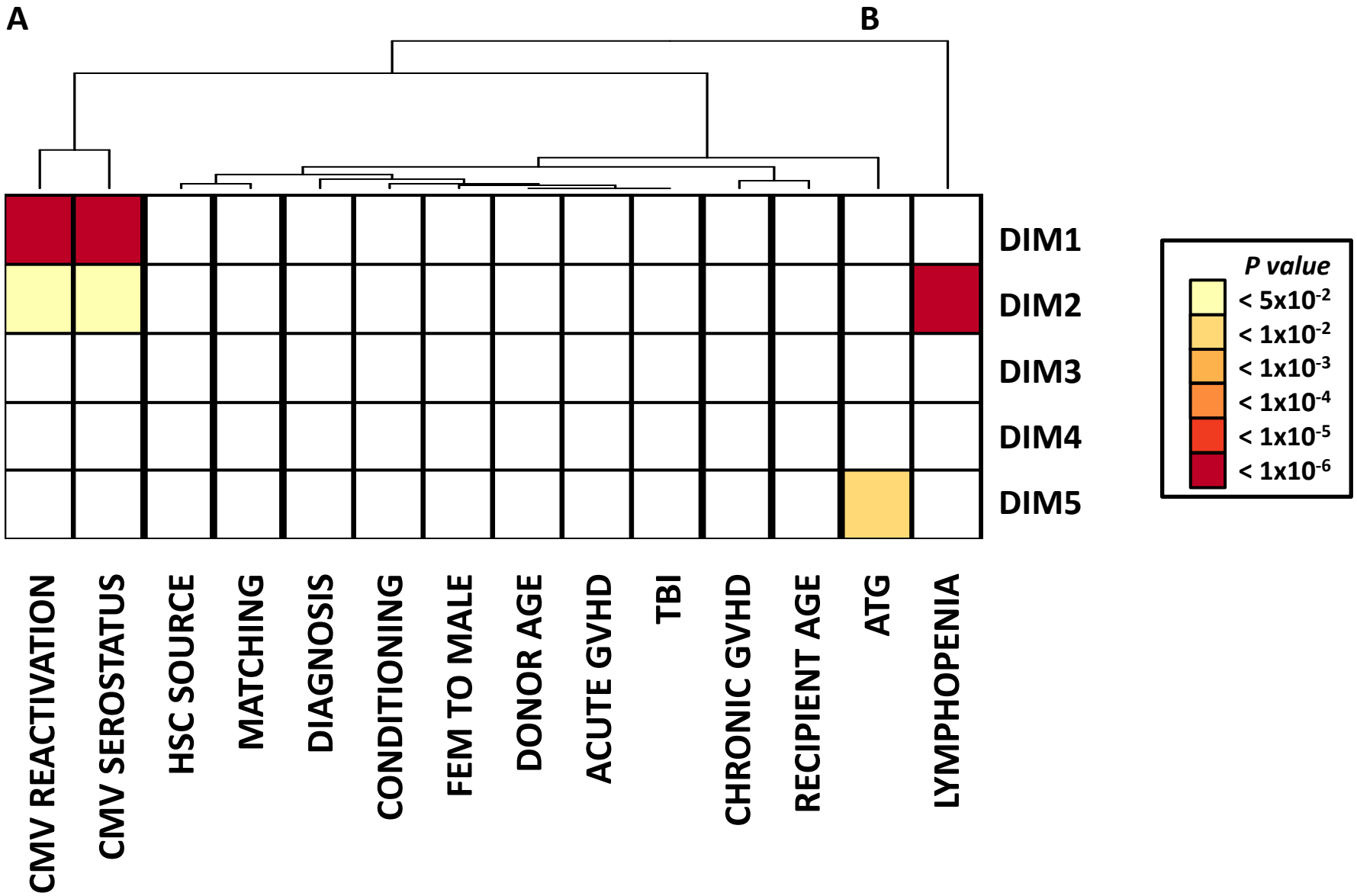
Itzykson et al. Supp. Figure 1





Itzykson et al. Supp. Figure 2

P<0.05



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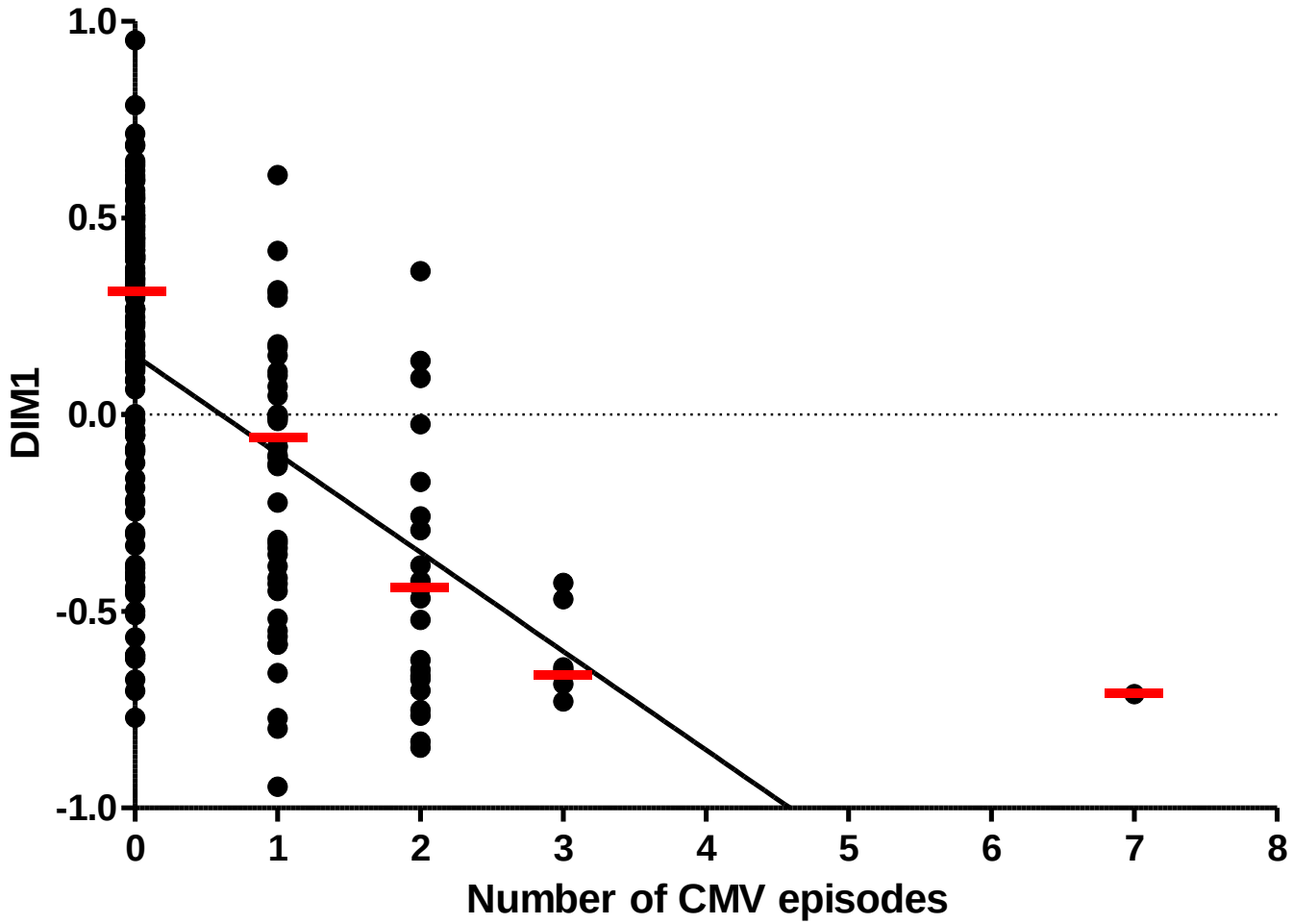
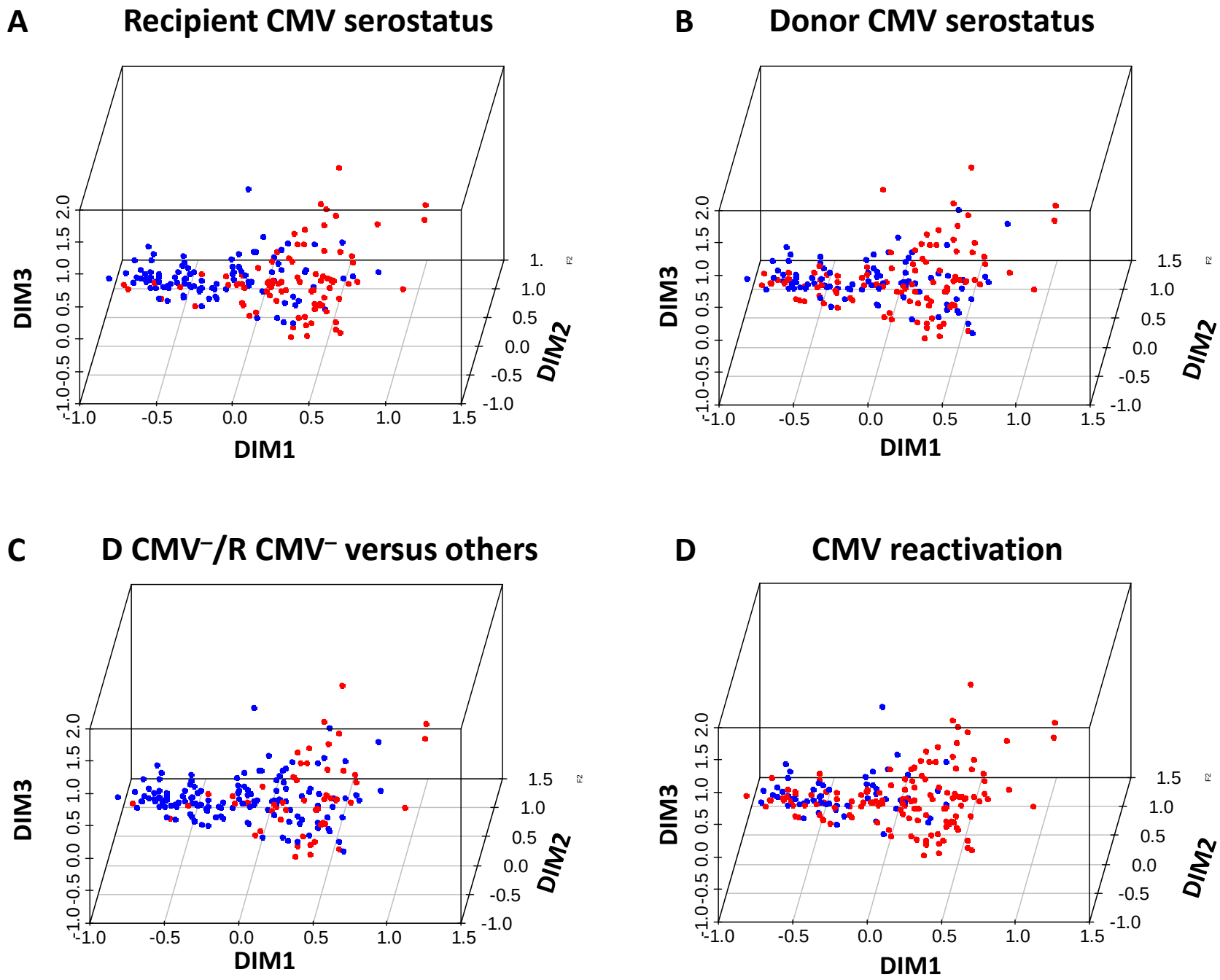


Itzykson et al. Supp. Figure 4



Itzykson et al. Supp. Figure 5